Recombined Egg Yolk Granules with Controllable Particle Sizes for Quercetin Delivery - A Structure-Activity Relationships Study

Teng Li  
Zhejiang Gongshang University

Junze Yao  
Zhejiang Gongshang University

David W. Everett  
AgResearch

Yilin Hou  
Zhejiang Gongshang University

Zhongshun Pan  
Zhejiang Gongshang University

Huanhuan Su  
Zhejiang Gongshang University

Yuying Fu (✉ webfu@126.com)  
Zhejiang Gongshang University

Research Article

Keywords: Recombined egg yolk granules, particle size, quercetin, stability, bioactivity.

Posted Date: May 25th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2954831/v1

License: ☺ This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Additional Declarations: No competing interests reported.
Recombined Egg Yolk Granules with Controllable Particle Sizes for Quercetin Delivery - A Structure-Activity Relationships Study

Teng Li¹; Junze Yao¹; David W. Everett¹,²,³; Yilin Hou¹; Zhongshun Pan¹, Huanhuan Su¹; and Yuying Fu¹*

¹ School of Food Science and Biotechnology, Zhejiang Gongshang University, Hangzhou, China.

² AgResearch, Palmerston North, New Zealand.

³ Riddet Institute, Palmerston North, New Zealand.

*Corresponding Author:

Yuying Fu, PhD, Professor

School of Food Science and Biotechnology, Zhejiang Gongshang University, Hangzhou, 310018, China.

Tel: +86-13857186960

E-mail addresses: webfu@126.com
Abstract

The effect of particle size on physicochemical stability, bioaccessibility, and bioactivity of bioactive compounds is important in the design of delivery systems. It is challenging to control the size of delivery systems without altering chemical composition. In the present study, the re-assembly of dissociated egg yolk granules was modulated by Ca$^{2+}$ to produce recombined granules with controlled hydrodynamic diameter ($D_h$). Quercetin was selected as a representative bioactive compound encapsulated within recombined granules (Gra-Que). Encapsulation efficiency and loading efficiency increased with an increase in $D_h$ until phase separation occurred at 250 µg/mL Ca$^{2+}$. The thermal and photochemical stability of Gra-Que increased as $D_h$ increased, whereas samples with the smallest $D_h$ demonstrated the highest storage stability. Gra-Que with an intermediate $D_h$ had the highest bioaccessibility due to a balance between the protection of quercetin and susceptibility to digestion. Smaller samples had higher bioactivity due to higher cellular uptake and greater susceptibility to digestion.

Key words: Recombined egg yolk granules; particle size; quercetin; stability; bioactivity.
1. Introduction

Natural herbal bioactive compounds, such as quercetin, curcumin, and apigenin, are attracting growing attention due to beneficial properties such as antioxidative and anticarcinogenic activities. Unfortunately, many herbal bioactive compounds are water insoluble due to their hydrophobic nature, leading to extremely low bioaccessibility and bioactivity. Therefore, much research has been directed towards developing delivery systems to improve the water solubility and functional performance of hydrophobic bioactive compounds. Hydrophobic bioactive compounds are often co-assembled with various compounds including small molecular weight surfactants, synthetic polymers, natural proteins, and/or polysaccharides [1, 2]. Anti-solvent, pH shifting, emulsification, and mechanical treatments have been commonly used to fabricate delivery systems [2, 3]. Hydrophobic bioactive compounds mainly interact with vehicles via hydrophobic interactions, resulting in nano- or micrometer-sized colloidal particles with improved bioaccessibility and bioactivity. Nevertheless, food-derived proteins are mostly used as vehicles for oral delivery systems due to structure-forming properties, affordability, biosafety, and biodegradability.

Elucidating the structure-activity relationships is of fundamental importance to rational design of delivery systems. Amongst the many physicochemical properties, particle size is a critical parameter with significant influence on colloidal, storage, thermal and light stability, and most importantly, bioaccessibility and bioactivity of encapsulated bioactive compounds [3]. Several studies have reported on the precise
control of protein particle size in delivery systems. Most studies introduce surfactants, salts, and biopolymers to regulate the self-assembly, and therefore the size of different proteins. The self-assembly of sodium casein can be modulated by a cationic surfactant dodecylammonium chloride [4]. By varying the surfactant aggregation states (monomeric or micellar) and concentration ratio of both components, soluble and insoluble complexes of nano- to micro-size can be obtained [4]. Choi and Zhong used different calcium-chelators to control the hydrodynamic diameter ($D_h$) of casein micelles [5]. The smallest $D_h$ was observed at an intermediate chelator concentration resulting from the balance of casein micelle dissociation and aggregation of dissociated caseins by an elevated ionic strength [5]. Zhou and co-workers employed chitosan and stearic acid conjugated chitosan to regulate the size and improve the water solubility of high-density egg yolk lipoprotein. The final complexes, with $D_h$ smaller than 100 nm, were shown to be promising nanoscale oral delivery vehicles for hydrophobic phytochemicals [6]. Incorporation of foreign molecules, especially biopolymers, alters the chemical composition of vehicles, making it difficult to isolate the effects of particle size. Mechanical treatments can reduce the size of large protein particles with minimal impact on chemical composition. High-intensity ultrasound and high-pressure treatments have been shown to dissociate egg yolk granules effectively [7, 8], but mechanical treatments are energy-consuming, and extra processes are still required for follow-up encapsulation. Therefore, a green one-step method to fabricate delivery systems with controllable particle size is needed for better understanding of the size
effects on physicochemical stability, bioaccessibility, and bioactivity.

Egg yolk can be separated into a supernatant plasma fraction and a sedimentary
granule fraction by a simple dilution and centrifugation process [9]. The granule
fraction accounts for 19-23 % yolk dry matter [10]. Yolk granules are natural protein
complexes mainly consisting of high-density lipoprotein and phosvitin. Under
physiological conditions, high-density lipoprotein and phosvitin self-assemble into
compact granules with $D_h$ ranging from around 300 nm to several micrometers [11].
The assembly of these granules is driven by the formation of Ca$^{2+}$ ion bridges between
phosphoserine amino acids in high-density lipoprotein and phosvitin [12, 13]. Given
the critical role of Ca$^{2+}$ in formation of granules, our previous study has demonstrated
that 0.1 wt% calcium chelators (Na$_5$P$_3$O$_{10}$, 2Na-EDTA, and C$_6$H$_5$Na$_3$O$_7$) can dissociate
sedimentary micrometer-sized granules into dispersible nanoparticles at neutral pH [11].
It was shown that the calcium chelator-induced dissociation is a physical process
without any changes in protein profiles of granules [11]. Further experiments have
demonstrated that calcium chelator-induced dissociation is reversible because
dissociated nanoparticles re-assemble to granules with the addition of Ca$^{2+}$. The $D_h$ of
the recombined granules can be precisely controlled by the amount of Ca$^{2+}$. It was also
shown that hydrophobic compounds can be encapsulated into granules in a reversible
self-assembly process [14]. Inspired by the findings above, we propose that recombined
granules are a good model to study the size effects on physicochemical stability,
bioaccessibility, and bioactivity of the delivery systems.
Quercetin is a dietary flavonoid, widely found in capers, black chokeberries, onions, tomatoes, and lettuce [2]. Various beneficial functions of quercetin have been identified, including antioxidative [15] and anti-inflammatory activity [15], anti-obesity [16], anticarcinogenic [17], antiviral [18], and antibacterial activities [19]. In addition to insolubility and low bioaccessibility [20], quercetin is not stable under common food sterilization conditions, such as thermal treatments and UV-light exposure [20, 21]. In this study, we manufactured a delivery system for quercetin using recombined egg yolk granules as vehicles. The \( D_h \) of the delivery system was precisely controlled by the amount of \( Ca^{2+} \) added. The aim of this research was to elucidate the effects of particle size on physicochemical stability, bioaccessibility, and bioactivity of encapsulated quercetin within a designed protein-based delivery system.

2. Materials and methods

2.1 Materials

Fresh eggs were purchased from a local grocery in Hangzhou, China. Quercetin (purity >97%) was purchased from Macklin Co. Ltd (Shanghai, China). Dulbecco's modified eagle medium (DMEM) (T11965084) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Special grade fetal bovine serum (FBS) was purchased from Zhongsheng Aobang Biological Technology (Beijing, China). HT-29 lines were purchased from the American Type Culture Collection (Manassas, VA, USA). HPLC grade acetonitrile was purchased from Anhui Tedia High Purity Solvent Co., Ltd. (Anqing, Anhui, China). Deionized water (≤2.04 \( \mu S/cm \) conductivity) was purchased
from the Renhe Water Purification Company (Hangzhou, Zhejiang, China) and was used throughout the experiment. All other reagents were of analytical purity.

### 2.2 Preparation of quercetin loaded recombined granules (Gra-Que)

Egg yolk granules were prepared according to a previous study [22]. Egg yolk granules were pre-dissociated with 2Na-EDTA, and excessive 2Na-EDTA was removed by dialysis [11]. As shown in Figure 1A, Gra-Que was prepared by an anti-solvent method [23]. Quercetin and CaCl$_2$ were co-dissolved in ethanol and added to dissociated granules dispersions (1.0 wt%) dropwise with constant stirring. The final quercetin in each sample was fixed at 0.5 mg/mL, whereas CaCl$_2$ concentrations ranged from 0 to 300 μg/mL. The mixtures were stirred for 3 h, then centrifuged at 2000 g for 10 min. Supernatants were collected as stable Gra-Que dispersions. Samples with CaCl$_2$ concentrations of 0, 100, 150, 200, 250 and 300 μg/mL were designated as Gra-Que0, Gra-Que1, Gra-Que2, Gra-Que3, Gra-Que4, Gra-Que5, respectively.

### 2.3 Encapsulation efficiency and loading efficiency

The encapsulation efficiency (EE%) and loading efficiency (LE%) of quercetin in Gra-Que was determined based on a method described previously [22]. The freshly prepared Gra-Que dispersions were diluted 100× with anhydrous ethanol and vortexed for 2 min. The mixture was centrifuged at 2000 g for 10 min, then the absorption of supernatant at 372 nm was recorded with a UV-Vis spectrophotometer (UV-2600, Shimadzu, Kyoto, Japan). The quercetin concentration was determined with a pre-established calibration curve. EE% and LE% were calculated using following equations:
\[
EE(\%) = \left( \frac{\text{weight of quercetin in the clear supernatant}}{\text{weight of total quercetin added}} \right) \times 100\% \quad (1)
\]

\[
LC(\%) = \left( \frac{\text{weight of encapsulated quercetin}}{\text{weight of Gra–Que}} \right) \times 100\% \quad (2)
\]

2.4 Hydrodynamic diameter (\(D_h\)) and \(\zeta\)-potential

The \(D_h\), polydispersity index (PDI), and \(\zeta\)-potential of the Gra-Que were determined with a Nano ZS dynamic light scattering system at 25°C (Malvern Instruments, Malvern, UK). Before the determination, samples were diluted with water to avoid multiple scattering effects.

2.5 Transmission electron microscopy

The morphology of dissociated granules, Gra-Que0, Gra-Que3, and Gra-Que5 were analyzed by transmission electron microscopy (TEM). Samples (10 µL, 2.0 mg/mL) was loaded onto 230-mesh carbon grills (Zhongjingkeyi Technology Co., Ltd., Beijing, China). Excess moisture was absorbed and removed with filter paper after 5 min. The morphology of samples was observed with a JEM-1400Flash TEM (JEOL Ltd, Japan) at 80 kV.

2.6 X-ray diffraction

The crystalline state of free quercetin, physical mixture of granules and quercetin, and Gra-Que3 were characterized with an X-ray diffraction (XRD) instrument (D8 Advance, Bruker, Germany). Scans were conducted with scattering angle (2θ) from 5° to 70° at a scan rate of 2 °/min.

2.7 Fourier transform infrared spectroscopy

Freeze-dried samples (2 mg) were mixed with 200 mg of dried KBr, then the
mixtures were pressed into thin tablets. Fourier transform infrared (FTIR) transmittance spectra over the range of 400 to 4000 cm\(^{-1}\) were collected with a Nicolet iS10 FTIR Spectrometer (Thermo Fisher Scientific). All spectra were obtained at a resolution of 4 cm\(^{-1}\) using 32 scans.

2.8 Intrinsic fluorescence spectroscopy

The interactions between granules and quercetin were investigated by intrinsic fluorescence spectroscopy based a published method [24]. Quercetin-ethanol solution was added to dissociated granule dispersions (5.0 mg/mL) to reach final quercetin concentrations ranging from 0 to 60 µM. The fluorescence intensity was measured with an FLS 980 fluorescence spectrophotometer (Edinburgh Instruments, Livingston, Scotland) at an excitation wavelength of 280 nm. The emission spectra between 300 and 400 nm were recorded. The slit width for excitation and emission was set to 2 nm.

2.9 Far-UV circular dichroism spectroscopy

Influence of quercetin loading on secondary structure of recombined granules was investigated by far-UV circular dichroism (CD) spectroscopy. The CD spectra in the far-UV (190–260 nm) region of the 0.5 mg/mL samples dispersed in water were scanned using a Jasco J-810 CD spectropolarimeter (Jasco Corp, Tokyo, Japan) at a scan rate of 100 nm/min. The thickness of the sample cell was 1.0 mm. All measurements were conducted at 25°C. The secondary structural contents of the different samples were analyzed using DichroWeb [25] with the CINTINLL algorithm [26].
2.10 Thermal, photochemical, and storage stabilities of Gra-Que

2.10.1 Thermal stability

Freshly prepared Gra-Que0, Gra-Que1, Gra-Que3, and free quercetin were dispersed in water and heated in 60, 75, and 90°C water baths for 30 min with constant stirring. After thermal treatments, 0.1 mL samples were diluted with 9.9 mL ethanol. The concentration of quercetin was measured using the method in section 2.3. Thermal stability was calculated as retention rate after treatments:

\[
\text{Retention rate (\%)} = \left( \frac{\text{concentration of quercetin after heating}}{\text{concentration of quercetin before heating}} \right) \times 100\% \quad (3)
\]

2.10.2 Photochemical stability

The photochemical stability of free and encapsulated quercetin was evaluated according to a method described previously [27]. Freshly prepared sample (Gra-Que0, Gra-Que1, Gra-Que3) and free quercetin were dispersed in water with constant stirring. Dispersions were exposed to 40 W UV-light irradiation. Quercetin concentrations were measured in samples treated with irradiation at 0, 30, 60, 120, 240, 360, and 480 min. Photochemical stability was calculated as retention rate at each sampling point:

\[
\text{Retention rate (\%)} = \left( \frac{\text{concentration of quercetin of each sampling point}}{\text{concentration of quercetin at 0 min}} \right) \times 100\% \quad (4)
\]

2.10.3 Storage stability

The freshly prepared Gra-Que0, Gra-Que1, and Gra-Que3 were stored at 4°C in the dark for 28 days. Quercetin concentrations were measured at 0, 1, 3, 7, 14, 21, and 28 day with the method described in section 2.3. Storage stability was calculated as retention rate at each sampling point:
Retention rate (%) \( = \left( \frac{\text{concentration of quercetin of each sampling point}}{\text{concentration of quercetin at 0 day}} \right) \times 100\% \) (5)

2.11 Bioaccessibility of quercetin evaluated by simulated gastrointestinal digestion

The bioaccessibility of free and encapsulated quercetin was determined by simulated gastrointestinal digestion according to a reported method with modifications [28]. Freshly prepared dispersions (20 mL, containing 0.3 mg/mL quercetin) were mixed with 20 mL simulated gastric fluid (containing 40 mg NaCl and 64 mg pepsin), and the pH of the solutions was adjusted to 2.0. Thereafter, the mixtures were incubated at 37 °C with constant stirring for 120 min. The pH of the mixtures was adjusted to 7.4, and 144 mg trypsin and 188 mg bile salt were added, followed by a 240-min incubation at 37 °C with stirring. After simulated digestion, mixtures were centrifuged (8,000 \( g \), 30 min), the supernatant was collected. The quercetin in the supernatant was measured using a C18 reverse-phase column (250 mm × 4.6 mm, Ultimate, Shanghai, China) in a high-performance liquid chromatography (HPLC) system (1260 Agilent, Agilent Corporation, Santa Clara, CA, USA). The mobile phase consisted of 0.1% acetic acid, 32% acetonitrile, and 67.9% HPLC water. The flow rate of the mobile phase was 1.0 mL/min. A UV-visible detector measured the absorbance of the samples at a wavelength of 372 nm. The injection volume of each sample was 20 \( \mu\)L. The temperature of the C18 column was maintained at 30 °C. The quercetin concentration in the supernatant was determined using a calibration curve constructed by dissolving different amounts of quercetin in acetonitrile.
2.12 Bioactivity of quercetin

2.12.1 Free radical scavenging activity

The antioxidant activities of the samples were evaluated using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity and 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) free radical scavenging activity assays. The DPPH radical scavenging activity of samples was determined according to a published method [22]. Free quercetin was dissolved in ethanol, and DPPH was dissolved in ethanol at 0.05 mM. The free and encapsulated samples were then mixed with 10.0 mL of DPPH solution to reach final quercetin concentrations of 2.5 and 5.0 µg/mL, respectively. The mixtures were vortexed and incubated in the dark for 30 min at 25 °C and the absorbance of the mixtures was measured at 517 nm. Pure ethanol was used as a control. The DPPH radical scavenging activity was calculated according to the following equation:

\[
\text{DPPH scavenging activity(\%)} = \left(\frac{A_{\text{control}}}{A_{\text{sample}}} - 1\right) \times 100\% \quad (6)
\]

where \(A_{\text{sample}}\) is the absorbance of the sample and \(A_{\text{control}}\) is the absorbance of the control. The DPPH radical scavenging activity of quercetin-free recombined granules was measured, and this value was subtracted from the value determined for the Gra-Que.

The ABTS radical scavenging activities of the samples were determined according to a published method [22]. ABTS was dissolved in water at 7.0 mM, and potassium persulfate (26.4 mg) was added to 40.0 mL ABTS solution with constant stirring at 25
13ºC in the dark for 16 h. The ABTS free radical solution was then diluted with PBS buffer (pH 7.4) to reach an absorbance of 0.7 at 734 nm (control). Free quercetin was dissolved in dimethyl sulfoxide (DMSO). Then, the DMSO-dissolved quercetin and Gra-Que solutions were added into 10.0 mL of diluted ABTS free radical solution. The final concentrations of quercetin were 0.8 and 1.6 µg/mL, respectively. After 6 min incubation at 25 ºC, the absorbance was measured at 734 nm. The ABTS scavenging activity was calculated according to the following equation:

\[
\text{ABTS scavenging activity (\%)} = \left( \frac{0.7 - A_{\text{sample}}}{0.7} \right) \times 100\% \quad (7)
\]

where \( A_{\text{sample}} \) is the absorbance of the sample at 734 nm. The ABTS radical scavenging activity of the quercetin-free recombined granules was measured and subtracted from the value obtained for the Gra-Que.

2.12.2 Anti-proliferation activity

HT-29 cells were cultured and harvested according to our previous protocols [22]. HT-29 cells were seeded in 96-well microtiter plates at a density of 1,000 cells per well in 100 µL DMEM (2.0% FBS). After incubated at 37ºC for 24 h, the original medium was removed. Then cells were incubated for 24 h with the medium containing 10–80.0 µg/mL DMSO-dissolved or encapsulated quercetin (Gra-Que0, Gra-Que1, and Gra-Que3). After incubation, the medium was aspirated, and 90 µL DMEM medium and 10.0 µL MTT (Shenggong Biological Engineering Co. Ltd, Shanghai, China) were added into each well. Cells were further incubated at 37 ºC for 4 h, then DMSO (100 µL) was added into each well. Microtiter plates were placed in a shaker for 30 min.
Finally, the absorbance at 570 nm was measured by microplate reader (Agilent Corporation). The percentage cell viability was then calculated using the following equation:

\[ \text{Cell viability}\% = \frac{A_{\text{treated}}}{A_{\text{control}}} \times 100\% \]  

where \( A_{\text{treated}} \) and \( A_{\text{control}} \) are the absorbance of cells with and without quercetin treatment.

### 2.13 Statistics analysis

Statistical analyses were carried out using a one-way ANOVA test followed by Duncan’s test (SPSS, Chicago, IL, USA, version 17.0). The significance levels were defined as probabilities of 0.05 or less. Values that do not share the same letter are significantly different.

### 3 Results and discussion

#### 3.1 Influence of \( D_h \) on quercetin encapsulation

As shown in Figure 1A, the EE% of Gra-Que increased with increased CaCl\(_2\), reached a maximum at 200 µg/mL CaCl\(_2\), then decreasing sharply at 250 µg/mL CaCl\(_2\). Without CaCl\(_2\), dissociated granules did not self-assemble to recombined granules, leading to less hydrophobic microenvironments for quercetin, therefore low EE%. The recombined granules showed 70-80 EE% at 100-200 µg/mL CaCl\(_2\), which is similar to or higher compared with synthetic polymers and other food proteins [19, 29, 30]. Further increases in CaCl\(_2\) to 250-300 µg/mL significantly reduced the EE%. Appearance of samples (1.0 mg/mL granules) is shown as the inset in Figure 1B.
Samples with 0-200 µg/mL CaCl$_2$ demonstrated good colloidal stability. The turbidity of Gra-Que dispersions gradually increased with an increase in CaCl$_2$. Phase separation was observed in samples with 250-300 µg/mL CaCl$_2$ due to excessive assembly (highlighted in the red rectangle), resulting in low EE% (quercetin was encapsulated in recombined granules but precipitated with Gra-Que4-5). Similarly, LC% increased with an increase in CaCl$_2$ over the concentration range of 0-200 µg/mL (Figure 1C). This result is expected because assembly of dissociated granules provide more hydrophobic microenvironments for quercetin. LC% of samples with 250-300 µg/mL CaCl$_2$ were not measured because Gra-Que completely precipitated after centrifugation.

The $D_h$ and PDI of different samples are shown in Figure 2A. The $D_h$ of dissociated granules is 70 nm, which agrees with our precious study [11]. Incorporation of quercetin led to larger $D_h$ even without CaCl$_2$, which is similar to the curcumin-loaded granules nanoparticles [22], indicating that hydrophobic small molecules can link dissociated granule fragments. A PDI of 0.3-0.4 indicated that samples had a narrow to intermediate $D_h$ distribution. The magnitude of the $\zeta$-potential of recombined granules was smaller than that of dissociated granules (Figure 2B), which is expected because some negatively charged groups were screened by Ca$^{2+}$ and were incorporated into internal space of recombined granules. The morphology of samples is shown in Figure 2C-F. Granules dissociated into nanoparticles after treatment with 2Na-EDTA (Figure 2C), which is in line with our previous study [11]. For Gra-Que, encapsulation of quercetin did not change the morphology of granules (Figure 2D-E). Micrometer-sized particles
were found in Gra-Que5, which explains the phase separation. In short, when the $D_h$ of recombined granules progressively increased, an initial increase of EE% and LC% was observed, followed by a decrease due to phase separation. A large $D_h$ of recombined granules is beneficial to quercetin encapsulation until the point where colloidal stability is destroyed.

### 3.2 Interactions between quercetin and granules

The XRD was performed to investigate the crystalline state of free quercetin, physical mixtures, and Gra-Que, which are shown in Figure 3A. Free quercetin had sharp diffraction peaks at 20 of 10.87°, 12.41°, 14.18°, 17.18°, 26.56°, and 27.45°, indicating the highly crystalline structure of free quercetin. The physical mixture also presented several crystalline diffraction peaks at 10.82°, 12.53°, and 27.39°. In contrast, no crystalline diffraction peaks were observed in Gra-Que, which confirms that quercetin was in the amorphous state.

Interactions between quercetin and granules were investigated by FTIR (Figure 3B). The characteristic bands of free quercetin were OH stretching (3307 cm$^{-1}$), C=O stretching (1664 cm$^{-1}$), aromatic ring stretching (1611 cm$^{-1}$), C=C stretching (1520 cm$^{-1}$), and C-O-C stretching (1260 cm$^{-1}$) [31, 32]. The dissociated granules spectrum contained characteristic peaks that were assigned to different functional groups [22, 33]: asymmetric and symmetric $-\text{CH}_2$ stretching of lipid chains (2924 and 2852 cm$^{-1}$), stretching of phospholipids, triglycerides, and cholesterol esters (1741 cm$^{-1}$), stretching of amide I and amide II (1653 and 1545 cm$^{-1}$), and symmetric stretching of PO$_2^{-1}$ (1089 cm$^{-1}$).
cm\(^{-1}\)). For Gra-Que, some of the characteristic peaks associated with crystalline quercetin were not observed, such as those at 1664, 1611, and 1521 cm\(^{-1}\), suggesting there was no free quercetin.

Binding between quercetin and granules was further investigated by measuring changes in the intrinsic fluorescence properties of granules. The changes in intrinsic fluorescence intensity of proteins were attributed to the changes in the microenvironments of tryptophan (Trp) and tyrosine (Tyr) residues induced by binding of small molecules [24, 34]. The fluorescence intensity of the nanoparticles progressively decreased with increasing quercetin concentration (Figure 3C), implying that Tyr and Trp were important binding sites for the quercetin molecules. Presumably, the hydrophobic quercetin molecules were bound to these non-polar amino acids through hydrophobic association.

The CD analysis was conducted to reveal the effect of quercetin binding on the secondary structure of recombined granules. Dissociated granules demonstrated a positive peak at 193.0 nm and a wide negative trough with a minimum at 217.4 nm in the CD spectrum (Figure 3D), indicating a large proportion of β-sheets [35]. Loading of quercetin only slightly shifted the minimum toward larger wavelengths, implying encapsulation did not significantly change the secondary structure. This was confirmed by the fitting of the CD spectrum (Table 1). β-sheet and unordered structures were two major components. The larger proportion of unordered structure is assigned to highly charged phosvitin at neutral pH [36]. Proportion of each component did not change
significantly before and after quercetin encapsulation.

3.3 Influence of $D_h$ on physicochemical stability of quercetin

Thermal stability is essential to food additives because thermal pasteurization or sterilization is needed for food safety. Thermal instability severely limits the applications of quercetin as a bioactive additive in food. As shown in Figure 4A, more than 50% free quercetin degraded after 30 min of treatments at 60, 75, and 90 ºC, which is in accordance with a previous study [37]. All encapsulated quercetin demonstrated good thermal stability at 60 ºC, indicating these delivery systems are stable under standard pasteurization conditions. However, Gra-Que0 lost the protective effect when the temperature increased to 75 ºC. Gra-Que3 was the only sample that retained 90% quercetin after heating at 90 ºC. It can be concluded that a larger $D_h$ is associated with better thermal stability of encapsulated quercetin. The improved thermal stability of encapsulated quercetin might result from the superior thermal stability of granules [38-40]. The high resistance to heat treatments is ascribed to the compact structure of granules due to numerous linkages of phosphocalcic bridges [39]. This is further supported by the finding that native granules have better thermal stability than dissociated granules [39]. Presumably, quercetin binds to hydrophobic domains and is incorporated into internal microenvironments of recombined granules. However, these hydrophobic domains may be exposed due to thermal denaturation, resulting in degradation of quercetin. Ca$^{2+}$-induced assembly improves structural stability of recombined granules, therefore, minimizes thermal denaturation and degradation of
quercetin. It should be noted that the size effects may vary depending upon vehicles. For example, thermal stability of quercetin in zein/fucoidan nanoparticles decreases with an increase in particle size [28].

Quercetin is susceptible to UV-light. More than 40% free quercetin degraded after a 500 min UV-light treatment (Figure 4B), which agrees with previous studies [29, 41]. Encapsulation significantly improved the photochemical stability of quercetin. Gra-Que0 retained 76% quercetin. Higher retention rates (90%) were found in Gra-Que1 and Gra-Que3, which implies that a large $D_h$ is beneficial to photochemical stability of quercetin. Without Ca$^{2+}$-induced assembly, some quercetin molecules might adsorb to the surface of Gra-Que0, leading to higher susceptibility to UV-light and lower photochemical stability [42]. Recombined granules might have more internal microenvironments for quercetin and hence could protect quercetin from UV-light more effectively.

The storage stability of delivery systems was evaluated over a 30-day storage period at 4 °C (Figure 4B). Maximum quercetin was retained Gra-Que0. Retention rate decreased with an increase in $D_h$, which is the opposite to thermal and photochemical stabilities. Sediments were observed in the Gra-Que1 and Gra-Que3 but not in Gra-Que0 (data not shown) after centrifugation. Recombined granules might further assemble to sediment large particles during storage, resulting in loss of quercetin and hence lower retention rates. Similar results have been reported in a soybean protein-based quercetin delivery system [43].
3.4 Influence of $D_h$ on bioaccessibility and bioactivity of quercetin

The bioaccessibility was evaluated by measuring the quercetin released after simulated digestion. Less than 3% quercetin was released for free quercetin and Gra-Que after simulated gastric digestion (Figure S1). It has been found that dissociated granules can re-assemble to micrometer-sized particles at pH 3.0 [11]. This re-assembly might protect Gra-Que from gastric digestion, inhibiting release of quercetin. Bioaccessibility of quercetin after simulated gastrointestinal digestion is shown in Figure 5. Less than 5% free quercetin was released. The low bioaccessibility of free quercetin was ascribed to low solubility during digestion [44]. Encapsulation significantly improved the bioaccessibility of quercetin. Bioaccessibility of the granules-based delivery system was similar to that of caseinate [45] and soybean protein [43]. The numerous phosphocalcic bridges make the granule structure very compact and weakly accessible to enzymes [10]. Gra-Que0 had the smallest $D_h$ and the largest specific surface area, which was most susceptible to digestive enzymes. This led to fast degradation of granules and reduced the protection of quercetin. In contrast, Gra-Que3 with the largest $D_h$ was weakly accessible to digestive enzymes, inhibiting the release of quercetin. Gra-Que1 with an intermediate $D_h$ had the highest bioaccessibility because Gra-Que1 achieved the best balance between digestibility and the protective effect.

3.5 Influence of $D_h$ on bioactivity of quercetin

The bioactivity of quercetin was evaluated by characterizing the antioxidant
properties using DPPH and ABTS free radical scavenging activity assays. In the DPPH
assay, the free quercetin was dissolved in ethanol, whereas in the ABTS assay it was
dissolved in DMSO. In general, the differences between organic solvent-dissolved and
encapsulated quercetin were small in both assays (Figure 6A-B), which suggests that
encapsulation did not decrease the free radical scavenging activity of the quercetin. The
effect of $D_h$ was not significant as samples with different $D_h$ presented similar
antioxidant properties ($p \leq 0.05$).

The bioactivity of quercetin was also evaluated by measuring the ability to inhibit
the proliferation of HT-29 cells. It has been found that quercetin can inhibit proliferation
of different cancer cell lines both in vitro and in vivo [46-48]. Quercetin exhibited dose-
dependent anti-proliferation activity on HT-29 cells in vitro (Figure 6C). Gra-Que0
demonstrated the strongest anti-proliferation activity, followed by Gra-Que1, whereas
the activity of Gra-Que3 was even weaker than that of DMSO-dissolved quercetin.
Therefore, Gra-Que with smaller $D_h$ exhibited higher bioactivity in the HT-29 cell
model in vitro. The bioactivity of quercetin is dominated by cellular uptake and
intracellular digestion. Nanoparticles uptake can be divided into an adhesion process
and a follow-up internalization process [49]. Size and surface charge density have been
shown as two important properties affecting nanoparticles uptake by cells. For
nanoparticles with similar surface charge densities, smaller ones are more prone to
cellular uptake as less driving force and energy are required in the cellular
internalization process [50]. In addition, Gra-Que0 was more susceptible to digestion
due to a lack of phosphocalcic bridges. Therefore, Gra-Que0, with the smallest $D_h$, demonstrated the highest anti-proliferation activity. But size effects might be dependent upon type of cells. Further studies are still needed to elucidate size effects on bioactivity with other cell lines.

4 Conclusions

Understanding the influence of size of delivery vehicles on physicochemical stability, bioaccessibility, and bioactivity of encapsulated bioactive compounds is important in the design of delivery systems. This study has shown that quercetin can be encapsulated into recombined granules. By modulating Ca$^{2+}$ concentrations, $D_h$ of resultant particles can be precisely controlled, ranging from 120 nm to micrometer-sized. EE% and LC% increased with an increase in $D_h$, but sharply decreased when colloidal stability was destroyed. Encapsulation was confirmed by XRD and FTIR, and quercetin was in an amorphous state. Intrinsic fluorescence analysis revealed the important role of hydrophobic interactions in binding of quercetin to recombined granules. The thermal and photochemical stabilities of Gra-Que increased as $D_h$ increased, whereas samples with smaller $D_h$ had higher storage stability. Gra-Que1 with an intermediate $D_h$ had the highest bioaccessibility due to good balance between the protective effect of quercetin and susceptibility to digestion. The anti-proliferation of HT-29 cell experiment revealed smaller samples had higher bioactivity, probably because these were more prone to cellular uptake and could be utilized by cells more easily. The overall results indicate that it is difficult for one sample to achieve the best
physicochemical stability, bioaccessibility, and bioactivity. Factors such as processing methods and storage conditions should be comprehensively taken into consideration to design granules-based delivery systems.

Abbreviations used

\(D_h\): hydrodynamic diameter; \(PDI\): polydispersity index; \(Gra\): dissociated granules; \(Gra-Que (0-5)\): quercetin loaded recombined granules with different \(\text{CaCl}_2\) and \(D_h\); \(EE\%\): encapsulation efficiency%; \(LC\%\): loading efficiency%; \(XRD\): X-Ray Diffraction; \(FTIR\): Fourier Transform Infrared Spectroscopy; \(CD\): circular dichroism; \(TEM\): transmission electron microscopy.

Acknowledgments

We thank Dr. Yumei Qin for her kind help in cell measurements.

Funding

This work financially co-supported by the National Natural Science Foundation of China 32172209 and 32201957.

Conflict of interest

The authors declare that they have no conflict of interest.
References

11. Y. Fu, J. Yao, H. Su and T. Li, Food Biophys. 17, 302-313 (2022).
27. J. C. Cuevas-Bernardino, F. M. A. Leyva-Gutierrez, E. J. Vernon-Carter, C.


C. Zhang, Y. Fu, Z. Li, T. Li and Z. Li, Food Chem. 346, 128963 (2020).


C. He, Y. Hu, L. Yin, C. Tang and C. Yin, Biomaterials 31 (13), 3657-3666 (2010).

Figure captions

Figure 1. (A) Schematic diagram of preparation of Gra-Que. (B) Encapsulation efficiency and (C) loading capacity of recombined granules induced by different amount of CaCl$_2$. Quercetin concentration was 0.5 mg/mL for all samples. Appearance of different samples (1.0 wt%) are shown as the inset in B.

Figure 2. (A) Hydrodynamic diameter ($D_h$) and polydispersity index (PDI) of Gra-Que with different concentrations of CaCl$_2$. (B) $\zeta$-potential of Gra-Que with different concentrations of CaCl$_2$. Transmission electron microscope images of, (C) dissociated granules, (D) Gra-Que0, (E) Gra-Que1, and (F) Gra-Que3.

Figure 3. (A) XRD spectra of free quercetin, physical mixture of quercetin and granules, and Gra-Que3. (B) FTIR spectra of free quercetin, dissociated granules, and Gra-Que3. (C) Fluorescence emission spectra of granules at various concentrations of quercetin. (D) CD spectra of dissociate granules (Gra), Gra-Que0, and Gra-Que2.

Figure 4. (A) Thermal stability, (B) photochemical stability, and (C) storage stability of free quercetin and encapsulated quercetin in Gra-Que with different $D_h$. Black, blue, and red letters in A represent significance tests for 60, 75, and 90°C.

Figure 5. Bioaccessibility of free quercetin and encapsulated quercetin in Gra-Que with different $D_h$ evaluated after simulated gastrointestinal digestion.

Figure 6. Bioactivity evaluated by (A) DPPH radical scavenging activity and (B) ABTS radical scavenging activity of free quercetin and encapsulated quercetin in Gra-Que with different $D_h$. Black and red letters represent significance tests for 2.5 and 5.0
µg/mL quercetin in A, and 0.8 and 1.6 µg/mL quercetin in B. (C) Bioactivity of free quercetin and encapsulated quercetin in Gra-Que with different $D_h$ evaluated by the ability to inhibit the proliferation of HT-29 cells. Black, red, and blue letters represent significance tests for 10, 40, and 80 µg/mL quercetin.
Table 1. Secondary structural contents from deconvolution of CD spectra of samples.

<table>
<thead>
<tr>
<th>Samples</th>
<th>α-helix (%)</th>
<th>β-sheet (%)</th>
<th>Turns (%)</th>
<th>Unordered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gra</td>
<td>10.8</td>
<td>35.8</td>
<td>21.8</td>
<td>31.6</td>
</tr>
<tr>
<td>Gra-Que0</td>
<td>9.6</td>
<td>37.0</td>
<td>21.6</td>
<td>31.8</td>
</tr>
<tr>
<td>Gra-Que2</td>
<td>9.4</td>
<td>37.0</td>
<td>22.1</td>
<td>31.5</td>
</tr>
</tbody>
</table>
Figure 1

- Encapsulation efficiency (%)
- CaCl$_2$ concentration (μg/mL)
- Loading capacity (%)
- CaCl$_2$ concentration (μg/mL)

A. Dissociated granules

B. Graph showing encapsulation efficiency with different CaCl$_2$ concentrations.

C. Graph showing loading capacity with different CaCl$_2$ concentrations.
Figure 2

A. Variation of $D_h$ (nm) with CaCl$_2$ concentration (μg/mL) for granules.

B. Variation of ζ-potential (mV) with CaCl$_2$ concentration (μg/mL) for granules.

C. Dissociated granules

D. Gra-Que0

E. Gra-Que3

F. Gra-Que5
Figure 3

A. X-ray diffraction patterns of Free Que, Physical mixture of Gra and Que, and Gra-Que3. The graph shows the intensity (a.u.) against 2θ (degree).

B. Transmittance (%) against Wavenumbers (cm⁻¹) for Gra, Gra-Que3, and Que. The graph highlights specific wavenumbers such as 3410, 1664, 1541, and 1089 cm⁻¹.

C. Intensity (a.u.) against Wavelength (nm) for various concentrations of Gra, Gra-Que0, Gra-Que2, and Gra-Que2. The graph shows the effect of different concentrations on the intensity.

D. CD (mdeg) against Wavenumbers (nm) for Gra, Gra-Que0, and Gra-Que2. The graph displays the CD values for different wavenumbers.
Figure 4

**A**

Retention rate (%) vs. temperature (60 °C, 75 °C, 90 °C) for Que, Gra-Que0, Gra-Que1, and Gra-Que3.

**B**

Retention rate (%) vs. UV treatment (min) for Que, Gra-Que0, Gra-Que1, and Gra-Que3.

**C**

Retention rate (%) vs. storage duration (days) for Gra-Que0, Gra-Que1, and Gra-Que3.
Figure 5

![Bar chart showing bioaccessibility (%) for Quercetin and Gra-Que0, Gra-Que1, Gra-Que3.](image-url)

- Bioaccessibility (%)
- Quercetin, Gra-Que0, Gra-Que1, Gra-Que3
- Bars labeled a, b, c, d
Figure 6

A. DPPH radical scavenging (%)
- Que concentration (2.5 μg/mL)
- Que concentration (5.0 μg/mL)

B. ABTS radical scavenging (%)
- Que concentration (0.8 μg/mL)
- Que concentration (1.6 μg/mL)

C. Cell viability (%)
- Quercetin concentration (μg/mL)
- DMSO-Que
- Gra-Que0
- Gra-Que1
- Gra-Que3

Legend:
- a, b, c indicate significant differences at p < 0.05.
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supportingdata.docx